

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 April 2001 (12.04.2001)

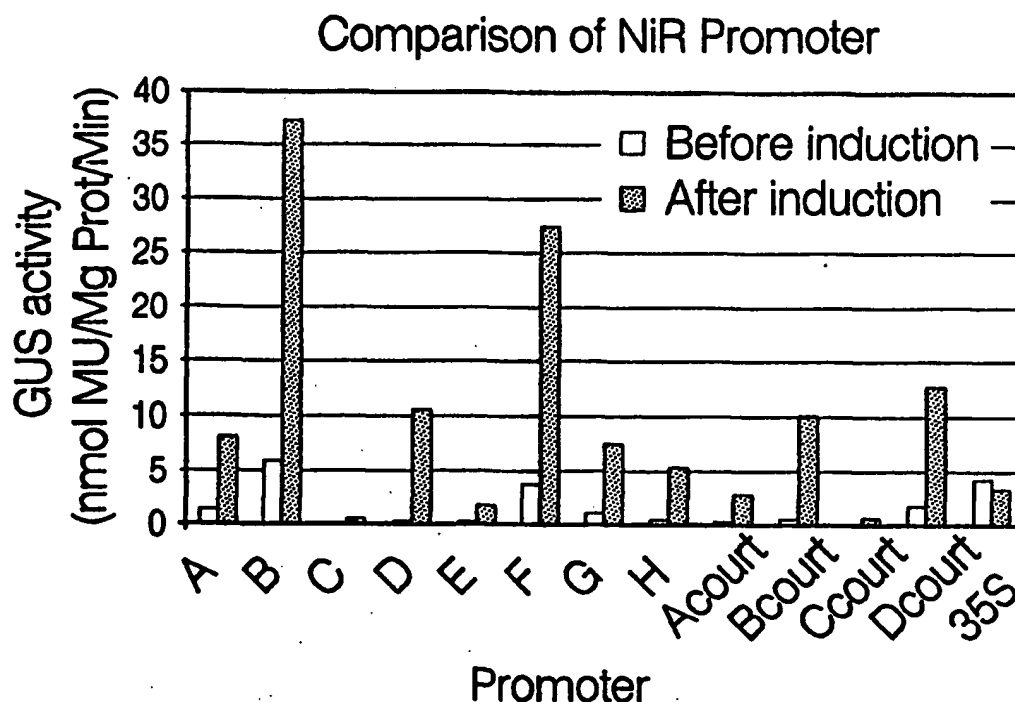
PCT

(10) International Publication Number
WO 01/25454 A2

- (51) International Patent Classification⁷: C12N 15/82, 9/02
- (21) International Application Number: PCT/CA00/01143
- (22) International Filing Date: 2 October 2000 (02.10.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/157,133 4 October 1999 (04.10.1999) US
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- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— Without international search report and to be republished upon receipt of that report.

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(54) Title: METHOD FOR REGULATING TRANSCRIPTION OF FOREIGN GENES



(57) Abstract: The present invention relates to a method of regulating the transcription of transgene in genetically-modified organisms. More specifically, the invention relates to the use of expression vectors harboring the coding sequence of a gene of interest under the transcriptional control of promoting sequences for which activity is regulated by the presence of nitrogen.



For two-letter codes and abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD FOR REGULATING TRANSCRIPTION OF FOREIGN GENES**BACKGROUND OF THE INVENTION****(a) Field of the Invention**

5 The invention relates to a method of regulating the transcription of transgene in genetically-modified organisms. More specifically, the invention relates to the use of expression vectors harboring the coding sequence of a gene of interest under the transcriptional control of promoting sequences for which activity is regulated by the presence of
10 nitrogen. Preferably, these constructs are used in transgenic leguminous plants (for example soybean, alfalfa, clover, birdsfoot trefoil, beans, peas, peanuts) where growth is not impaired by lack of mineral nitrogen, and in which induction of expression could be performed at any given time during development, through the addition of a suitable nitrogen source. In a
15 broader perspective, the invention could be used to induce expression of any given transgene through the addition of any nitrogen source, provided that the organism can be grown adequately in the absence of this nitrogen inducer; as an example within the plant kingdom, duckweed (*Lemna minor*) can adapt to grow either on nitrate or ammonium as nitrogen
20 source; transgenic duckweed could therefore be grown on nitrate as a sole nitrogen source and expression of the transgene triggered by the addition of ammonium, provided that the cassette contains a promoter from a native gene for which expression is turned on by the addition of ammonium. The invention therefore provides a means of regulating the
25 expression of a transgenic trait in any organism through the addition of various nitrogenous inducer.

(b) Description of Prior Art

 Nitrogen is a molecule essential to life. All living organism need nitrogen in order to synthesize amino acids, the building blocks of
30 proteins, and nucleotides, the building blocks of nucleic acids. It is Ammonium nitrate is the preferred form of mineral nitrogen provided to crops in the form of fertilizer. Nitrate-nitrogen is first reduced to nitrite and then to ammonium through the activity of a metabolic pathway common to most herbaceous plants. Depending on the species, part or all of the
35 absorbed nitrate will move to leaf cells through the xylem before it is

reduced to ammonium. Ammonium, or other reduced forms of nitrogen are also absorbed (although usually at lower rates) by the root system but their assimilation does not require reduction. These newly absorbed ammonium or ammonium-containing molecules join the endogenous pools in the cells which is formed by ammonium cycling through amino acids and other nitrogenous molecules. Some species do not metabolize nitrate-nitrogen easily and therefore cannot rely on nitrate as sole nitrogen source; many coniferous species fall into this latter category. Legumes and other symbiotic plant species form a third large class of nitrogen user within the plant kingdom; they form a metabolic alliance with a microbial organism through which they can fix gaseous nitrogen. This reduced nitrogen is used efficiently by the plant for growth, and therefore, these crops can develop independently of the availability of mineral nitrogen in the soil.

Many microbes and wild plant species will adapt extensively to availability of nitrogen sources and can therefore complete their life cycle in the absence of one molecular form of nitrogen, which they could use exclusively and efficiently if available in another growing environment. As for most assimilatory pathways, nitrogen assimilation is tightly regulated in cells. As an example, the expression of genes encoding nitrate reductase (NaR) and nitrite reductase (NiR), which are responsible for the reduction of nitrate to ammonium, has been extensively described in various microbial and plant species (for a review, see Mifflin and Lea, Books 5 and 12, in *The Biochemistry of plants*). Although nitrate is not the only regulatory molecule involved in the control of NaR and NiR expression, its presence is essential to initiate the cascade of transduction that eventually leads to sustained transcription and translation of these genes. It has been shown that expression of NaR and NiR genes is repressed in leguminous plants when they are grown in the absence of mineral nitrogen

NiR promoters have been characterized in some plant species (Back et al., 1991, *Plant Molecular Biology* 17:9-18; Sander et al., 1995, *Plant Molecular Biology* 27:165-177). Inducibility of these promoters have also been characterized using marker genes in transgenic plants, where it

was shown that availability of nitrate is required for full activation of transcription.

Assimilatory pathways for other nitrogen sources have also been described, and promoters for genes involved in some of these pathways have also been characterized.

Genetic transformation of microbes have been used for more than 15 years to produce useful recombinant molecules, and applications in the pharmaceutical, cosmaceutical and dermaceutical industries are being currently exploited. This technology has expanded from microbes to plants and animals in the last ten years with the development of techniques required to adapt this general concept to complex eukaryotic organisms. Basically a gene encoding for a protein of interest or a gene encoding for an enzyme responsible for a modification of a metabolic pathway that leads to a molecule of interest, is linked in an appropriate fashion to cis-and trans-acting regulatory sequences, and transferred to a target cell where it is incorporated in the molecular machinery (in a transitory or stable fashion). The transgenic cell, or a tissue or organism regenerated from the transgenic cell will then perform transcription and translation of the transgene and therefore be enabled to accumulate the protein of interest or to perform the new metabolic reaction through the activity of the enzyme of interest.

The emerging industry of molecular farming (production of recombinant molecules in animals or crops) is one of the most promising industry of the coming century. Its promise is to provide safe and renewable molecule factories for the industry. Among the applications that are currently developed are the production of low-cost monoclonal antibodies for therapeutic and diagnostic uses, the production of unlimited amounts of hormones, cytokines and other bio-active molecules for the treatment of chronicle or lethal diseases, the production of bio-safe substitutes for various blood components, the production of unlimited amounts of processing enzymes for the food and pulp industry, the production of low-cost enzymes for waste treatments, and the production of safe bio-active molecules for the cosmetic industry.

Limitations to the application of this technology has often come from the inability of transgenic organisms to accumulate adequate

amounts of the recombinant product, as a result of low transcription rates, improper splicing of the messenger, instability of the foreign mRNA, poor translation rates, hyper-susceptibility of the recombinant protein to the action of endogenous proteases or hyper-susceptibility of the recombinant organism to the foreign protein which result in improper and limited growth or in the worst cases, in strong deleterious effects to the host organism. Inadequacy of production level has a direct impact on the development of applications when profit margins are narrow, or when treatment and/or disposal of residual matter causes bio-safety or environmental problems.

Improvement of the accumulation level of the desired recombinant product thus appears to be one critical factor that warrants commercialization of many applications of molecular farming.

The use of inducible promoters has been proposed, and in some instances used successfully, to counteract the combined effect of all the above-mentioned factors. Strong inducible promoters may succeed in generating high ephemeral transcription rates which result in high transitory accumulation of foreign mRNA and translational product. As a result, when inducibility of expression is paired with adequate synchronized protein recovery procedures, the yield per unit obtained is higher than with the use of constitutive expression.

Several expression cassettes harboring inducible promoters have been developed for microbial production systems, and some are currently available for research purposes. Some inducible promoters are currently used in plant (wound inducibility) or animal (specificity to cells of the mammary glands, PPL) systems, although none reported are using low-cost and bio-safe chemical inducers such as nitrate salts.

It would be highly desirable to be provided with a method of regulating the transcription of transgene in genetically-modified organisms.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a method of regulating the transcription of transgene in genetically-modified organisms.

Another aim of the present invention is to provide the use of expression vectors harboring the coding sequence of a gene of interest under the transcriptional control of promoting sequences for which activity is regulated by the presence of nitrogen.

5 The present invention relates to the use of a nitrogen-inducible expression cassettes for the controlled expression of foreign genes in plants. It will be shown from the following description that isolating such a regulatory sequences can be performed so that when cis-acting sequences are appropriately associated to the open reading frame of a
10 gene of interest, its transcription can be controlled by the addition of specific nitrogen sources.

 In one aspect of this invention, the targeted system uses leguminous plant species, so that constructs containing a nitrate-inducible promoter will be maintained transcriptionally low throughout the growth
15 period if the transgenic plant is maintained on a nitrate-free medium, thus allowing the development of the plant biomass without interference from the transgenic trait. Upon addition of nitrate to the growth medium, transcription will be induced in a relatively large proportion of the biomass over the following days. Optimization of induction time and protein
20 accumulation will then be performed in order to maximize recovery of the desired recombinant product.

 Although the following description will make clear that this invention can be easily adapted to nitrate induction on nitrate-deprived transgenic leguminous plants, it should be remembered that this general
25 concept can also be applied to the development of other production systems, making profit of the wide variety of nitrogen assimilation systems in the microbial, plant and animal kingdoms.

 In one other aspect of this invention, nitrogen inducibility can also be used to maximize protein production in organisms which do not
30 perform nitrogen fixation through symbiotic association, but that can use variable sources of nitrogen (reduced or oxidized) for growth, and thus possess the ability to develop adequately while one of their nitrogen assimilation pathway is inactive due to lack of one nitrogenous substrate in the growing environment. Using an expression cassettes that controls
35 the transcription of any gene in this inactive pathway in order to drive the

expression of a gene of interest in such an organism, will allow for inducible expression of the transgenic trait upon addition of the previously lacking nitrogenous compound. As an example, duckweed is a plant species that can grow alternately on nitrate or ammonium; this invention
5 could be used to develop an expression cassette harboring an ammonium-inducible promoter appropriately linked to a gene of interest so that the induction would be performed on nitrate-grown transgenic duckweed plants.

In accordance with the present invention there is provided a
10 method for regulating transcription of a foreign gene in transgenic organisms, comprising the steps of:

- a) preparing a transgenic organism using an expression construct consisting of at least a nitrogen-inducible promoter having a sequence selected from the group consisting of SEQ ID NOS: 1
15 to 13 and functional fragments and derivatives thereof, and an ORF of a gene, wherein said promoter is operationally located with respect to said gene for expression of said gene.

In accordance with a preferred embodiment of the present invention, the method of may further comprise the step of regulating
20 transcriptional expression of said gene by addition or removal of a nitrogen inducer.

In accordance with a preferred embodiment of the present invention, there is provided a method for regulating transcription of a foreign gene in transgenic organisms comprising:

- 25 a) preparing an expression construct consisting of at least a nitrogen-inducible promoter with or without cis-acting sequence, an ORF of a gene, and a polyadenylation signal end site at the 3'end of said construct, wherein said promoter is operationally located with respect to said gene for expression of said gene
30 and modulated for transcriptional expression of said gene by addition or removal of a nitrogen inducer;
- b) sub-cloning the construct of step a) into a suitable transfection vector for said organism;
- c) transferring said vector into DNA of said organism or a cell
35 thereof; and

- d) selecting for transgenicity on a suitable medium.

In accordance with a preferred embodiment of the present invention, the method of may further comprise the steps of:

- 5 e) introducing the vector into a suitable *Agrobacterium tumefaciens* strain;
- f) using the *Agrobacterium* strain of step a) to transfer T-DNA into a plant cell;
- 10 g) selecting for transgenicity of said plant cell on a suitable medium;
- h) regenerating embryos or plantlets from said transgenic cells; and
- i) growing mature plants from said regenerated embryos.

15 In accordance with a preferred embodiment of the present invention, the cis-acting sequence may be isolated from 5' upstream region of an expressed Nir gene in alfalfa.

In accordance with a preferred embodiment of the method of the present invention, the promoter has the sequence set forth in SEQ ID NO:1 to 13 and functional fragments and derivatives thereof.

20 In accordance with a preferred embodiment of the present invention, the organism is a plant, more preferably a dicotyledonous plant.

In accordance with a preferred embodiment of the present invention, the organism is alfalfa or tobacco.

25 In accordance with a preferred embodiment of the present invention, the nitrogen inducer is nitrate.

In accordance with a preferred embodiment of the present invention, the DNA transfer method is any suitable transfer method including DNA bombardment, electroporation, PEG-mediated DNA transfer and whiskers, among others.

30 In accordance with a preferred embodiment of the present invention, the expression construct comprises at least a nitrogen-inducible promoter and at least one cis- or trans-acting elements.

35 In accordance with a preferred embodiment of the present invention, the organism is a plant, a fungus, a bacteria, a yeast or an animal.

In accordance with a preferred embodiment of the present invention, the promoter or cis-acting sequence is isolated from the 5' upstream region of any gene involved in a nitrogen assimilatory pathway.

5 In accordance with a preferred embodiment of the present invention, the promoter or cis-acting sequence is isolated from the 5'upstream region of any gene for which transcription is modulated by availability of a given nitrogen source.

10 In accordance with a preferred embodiment of the present invention, the promoting or cis-acting sequence is any sequence for which transcriptional activity is regulated by addition or removal of any nitrogen source in or from any living organism's environment.

In accordance with a preferred embodiment of the present invention, the organism from which the promoter or cis-acting sequence is isolated from is any plant, fungus, yeast, bacteria or animal.

15 In accordance with a preferred embodiment of the present invention, there is provided a promoter for promoting transcription of a foreign gene in transgenic organisms, which comprises a nitrogen-inducible promoter with or without cis-acting sequence for expression of said gene and adapted to be modulated for transcriptional expression of
20 said gene by addition or removal of a nitrogen inducer.

Preferably, the promoter has a sequence selected from the group consisting of SEQ ID NOS: 1 to 13 and functional fragments and derivatives thereof.

25 In accordance with a preferred embodiment of the present invention, there is provided a terminator allowing expression of a foreign gene in transgenic organisms being used in combination with a promoter, which comprises a polyadenylation signal end site for insertion at the 3'end of said gene, wherein said terminator is operationally located with respect to said gene and said promoter and thereby allows expression of
30 said gene.

Preferably, the terminator has a sequence selected from the group consisting of SEQ ID NOS: 14 to 16 and functional fragments and derivatives thereof.

35 For the purpose of the present invention the following terms are defined below.

The expression "functional fragments or derivatives thereof" is intended to mean any derivative or fragment of sequences SEQ. ID. NOS:1-16 which allow for an equivalent level of expression of a foreign gene as the promoter of the present invention set forth in SEQ. ID. NOS:1-13 or as the terminator of the present invention set forth in SEQ. ID. NOS:14-16.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates GUS expression level using promoter Nir (SEQ ID NOS:2-13) and terminator NOS in the leaves of transgenic tobacco plants before (empty columns) and after (filled columns) nitrate fertilization of the plants. Tobacco plants were transformed with full-length and deletions of the alfalfa NiR promoter and NOS terminator functionally positioned to control transcription and termination of the GUS reporter gene, as described in Methods. GUS activity was measured according to Jefferson et al. (1987, *EMBO J.* 13:3901-3907) prior and after induction by nitrate.

Fig. 2 illustrates GUS expression level using promoter Nir (SEQ ID NOS:2, 3, 5-6) and terminator Nir (SEQ ID NOS:15-16) in the leaves of transgenic tobacco plants before (empty columns) and after (filled columns) nitrate fertilization of the plants. Tobacco plants were transformed with full-length and deletions of the alfalfa NiR promoter and NiR terminator functionally positioned to control transcription and termination of the GUS reporter gene, as described in Materials and Methods below. GUS activity was measured according to Jefferson et al (1987, *EMBO J.* 13:3901-3907) prior and after induction by nitrate.

Fig. 3 illustrates GUS expression level using promoter NiR and terminator NOS in the leaves of nodulated transgenic alfalfa plants before (empty columns) and after (filled columns) nitrate fertilization. Alfalfa genotype 11.9 was transformed with constructs GC2-E, GC2-B, DC1-D and 35S functionally positioned to drive expression of reporter gene GUS, as described in Khoudi et al (1997, *Gene* 197:343-351). Following regeneration, transgenic plants were transferred to sterile vermiculite and inoculated with *Rhizobium* strain Balzac (Nitragin). Plants were allowed to grow for 3 weeks with repeated additions of nitrate-free Hoagland's

5 solution; GUS activity was then measured in first fully expanded leaves as described by Jefferson et al. (1987, *EMBO J.* 13:3901-3907). Plants were then fertilized with 40 mM nitrate for two days. GUS activity was again measured in first fully-expanded leaves. Data shown herein are ratios between post- and pre-nitrate induction GUS specific activities.

DETAILED DESCRIPTION OF THE INVENTION

10 Following is a detailed description of the method used to generate transgenic tobacco and alfalfa lines that can be modulated in their expression of a reporter gene. It should be remembered that variations could be brought to the method by which nitrogen-inducible promoters could be isolated, by which they could be linked to ORFs in the construct used for expression in plants, by which different cis- and trans-acting elements of the constructs are used and spatially arranged, by
15 which the inducibility by nitrogen is demonstrated and used, while remaining within the scope of this invention.

In this embodiment, a NiR cDNA strand was first isolated from alfalfa using RT-PCR with primers deduced from a consensus plant NiR sequence. This cDNA stretch was then used either to perform
20 upstream/downstream genome walking. The NiR promoter region and deletions, the 5'UTR and the NiR terminator were then positioned functionnally to control transcription and terminaison of reporter gene GUS. These constructs were inserted into suitable expression vectors for DNA bombardment onto tobacco and alfalfa leaves and for *Agrobacterium*
25 mediated DNA transfer as described by Desgagnés et al. (1995, *Plant Cell Tissue Organ Cult.* 42:129-140). These two DNA transfer methods were used to demonstrate that expression of the reporter gene can be modulated by addition or removal of nitrate in the growing medium.

Materials and Methods

Biological material

E. coli strain DH5- α was used to perform all cloning steps. Cold resistant alfalfa genotype 11.9 was used for all experiments including

stable transformation using *A. tumefaciens* infection (Desgagnés et al. (1995, *Plant Cell Tissue Organ Cult.* 42:129-140).

Isolation of total RNA

5 Total RNA was extracted using a hot phenol method essentially as described by de Vries et al. (1988, B6 page 1, In: Gevin SB and Shilperoot RA editors, *Plant Molecular Biology Manual*, Dordrecht: Kluwer Academic Publisher).

10 RT-PCR

RT-PCR was used to produce a DNA fragment corresponding to one abundant NiR mRNA molecular species from leaf total mRNA. A conserved region was first identified from 5 public plant NiR ORFs, namely Genbank sequences #AB006032 (*Arabidopsis* Nir mRNA), #
15 X66145 (*Tobacco* partial Nir mRNA), #U10419 (*Bean* complete Nir cds), #X07568 (*Spinach* Nir mRNA), and #U90429 (*Glycine max* Nir complete cds). Degenerated oligonucleotides were deduced from two conserved regions, namely Nir5 - 5' GATATTGATGTTAGACTCAAGTGGC 3' (SEQ ID NO:17), at the 5' end and Nir3 - 5' CACYSATTCCACTTCCTWGGC 3'
20 (SEQ ID NO:18), at the 3' end of the coding strand. A reverse transcription reaction was first performed with 200 units of M-MLV reverse transcriptase (RT) for 1 hour at 37°C using 1 µg of total leaf RNA, 4 mM dNTP (1 mM each), 5 µM random hexamer primers in a 1X M-MLV-RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3mM MgCl₂). The PCR
25 reaction was performed in a Perkin Elmer Cetus GenAmp PCR system 9600 (EG&G, Wellesley, MA), using 2.5 units of Taq DNA polymerase, 2 µM Nir5 primer, 2 µM Nir3 primer, 800 µM dNTPs (200 µM each) in a 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 2 mM MgCl₂). The cycling program used was: an initial 4 min at 94°C, 30 cycles of 1 min at
30 94°C, 30 sec at 55°C, and 3 min at 72°C. An extension period of 7 min at 72°C was included in the program.

DNA sequencing

DNA sequencing was performed as described by Sanger et al. (1977, *P.N.A.S. USA*, 74:5643-5647).

5

Genome walking

Walking upstream of the Alfalfa Nir cDNA fragment cloned from the RT-PCR reaction was performed using the Universal Genome Walker Kit from Clontech Laboratories (Palo Alto, CA) (Cat. #1807-1). The NiR-specific custom primers used to amplify sequences upstream of the coding sequence were:

10

Nir1106r - 5' TTGTCACATCAGCACATCCGTCTTTGC 3' (SEQ ID NO:19), and

15

Nir1061r - 5' TCGCCAAGTATCTTGTTTGAGCACTTG 3' (SEQ ID NO:20).

The amplified 3775 bp fragment was subcloned into pGEM-T Easy vector (Promega, Madison, WI) (Cat #A1360) for further analysis. The resulting plasmid was named pGNir4c.

The downstream walking was performed as the upstream walking using the following NiR specific primers:

20

Nir1c - 5' ATGTCTTCCTTCTCAGTACGTTTCCTC 3' (SEQ ID NO:28), and

Nir138c - 5' CAAGTTGATGCATCAAGGTGGGAGCCTAGA 3' (SEQ ID NO:29).

25

The amplified 3508 bp fragment was subcloned into pGEM-T easy vector (Promega, Madison, WI) (Cat #A1360) for further analysis. The resulting plasmid was named pGN3'1.

Construction of expression cassettes and vectors

The cassettes for expression analysis using the GUS reporter gene were assembled as follows. A promoterless GUS cassette was digested from pBI101 with HindIII and EcoRI, and was inserted into the HindIII and EcoRI sites of the pUC19 polycloning site. The resulting plasmid was named pBI201 and was used for further constructs. The Nir upstream sequences were PCR amplified using the AP2 primer from the Universal Genome Walking Kit as upstream positioned primer, and either one of custom-designed downstream primers ending with a SmaI

35

restriction site. The 4 primers were positioned either in the 5' UTR region of the gene (Nir-23r-Sma-5'AGAGCCCGGGAGAAGAGAGTGTGTTTG3' (SEQ ID NO:21)), at the end of the transit peptide coding sequence (Nir51r-Sma - 5' TTCTCCCGGGGGACGAGAGATGGATGGT 3' (SEQ ID NO:22)), 50 bp after the transit peptide coding sequence (Nir103r-Sma - 5' TTCTCCCGGGGTTGAA-ACAGGTGCAACTGA 3' (SEQ ID NO:23)), and 100 pb after the transit peptide coding sequence (Nir158r-Sma - 5' TTCTCCCGGGTAACCATCTTTTCCTCA 3' (SEQ ID NO:24)) Amplification was performed under standard conditions with pGNir4c plasmid as template.

The amplified fragments were digested with specific restriction enzymes in order to produce 5' deletions of the Nir promoter. The pNir3k-23 was produced by digesting the fragment previously amplified by AP2 and Nir-23r-Sma primers with XmaI, and inserting the resulting fragment into pBI201 previously digested with XmaI. A similar strategy was used to produce the pNir3k51, pNir3k103, and pNir3k158 plasmids except that the downstream primers used were Nir51r-Sma, Nir103r-Sma, and Nir158r-Sma, respectively. The pNir2.2k-23 was produced from a SmaI-BglII digestion of the AP2 - Nir-23-Sma amplified fragment inserted into the pBI201 previously digested with SmaI and BamHI. The same strategy was used to produce the pNir2.2k51, pNir2.2k103, and pNir2.2k158 plasmids except that the downstream primers used were Nir51r-Sma, Nir103r-Sma, and Nir158r-Sma, respectively. Fidelity and orientation of the insertions were verified by digestion with restriction enzymes. These deletion fragments were ligated to the 5'terminus of the GUS reporter gene in pBI201, and used for transitory expression studies using DNA bombardment. Upon identification of the adequate deletion fragments, they were sub-cloned into a binary plant expression vector such as pBI101 (Clontech).

For the construction of the cassettes containing the NiR terminator downstream of the GUS gene in addition to the NiR promoter, the following NiR specific primers were used:

Nir2514c-Sac - 5' AGAAGAGCTCAGTATATAGGTATTTGGTGA 3'(SEQ ID NO:30)

Nir2728c-Sac - 5' AGAAGAGCTCTTGTACATTTCGATAAGTCA 3' (SEQ ID NO:31)

Nir3029r-Eco - 5' AGAAGAATTCGTTTTCCCGATACTTCAACT 3' (SEQ ID NO:32)

5 A 617 bp terminator fragment was PCR amplified using the primers Nir2514c-Sac and Nir3029r-Eco, and a 503 bp terminator fragment was PCR amplified using the primers Nir2728c-Sac and Nir3029r-Eco. The fragments obtained were digested with SacI and EcoRI and inserted into the plasmids containing the NiR-GUS constructs after
10 deletion of the NOS terminator between the SacI and EcoRI sites.

These recombinant plasmids were used for stable integration through *A. tumefaciens* infection as described below.

15 ***Agrobacterium*-mediated DNA transfer and regeneration of transgenic alfalfa lines**

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation as described in Khoudi et al (1997, *Gene* 197:343-351). Selected *Agrobacterium* strains were then co-cultivated with leaf disks from genotype C5-1 for 4 days in the absence
20 of selection pressure (kanamycin). Following this incubation period, leaf disks were washed and pampered, and then allowed to form calli onto medium B5H. Calli were then transferred for 21 days on SH medium for embryo induction and for 28 days on BOi2Y for embryo development. Torpedo-shaped embryos were removed from BOi2Y and placed on MS
25 medium for regeneration. Kanamycin was present in all cultivation medium except for co-cultivation and regeneration on MS. This method is described in length in Desgagnés et al (1995, *Plant Cell Tissue Organ Cult.* 42:129-140). Rooted plantlets were grown to maturity in the greenhouse. Integration of the transgene was verified by PCR
30 amplification of a NiR-GUS fragment from genomic DNA. The primers used were:

Nir-102c - 5' CACACTTCTTCACTCACCTCTCAA 3' (SEQ ID NO:25)

Nir-2016c - 5' ATCTAGGAGGGGCAGACATTG 3' (SEQ ID NO:26)

GUS228r - 5' TCGGTATAAAGACTTCGCGCTGAT 3' (SEQ ID NO:27)

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***Agrobacterium*-mediated DNA transfer and regeneration of transgenic tobacco lines**

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation as described in Khoudi et al (1997, *Gene* 197:343-351). Selected strains were co-cultivated with leaf disks for 2 days on MS medium without kanamycin. After this period, the explants were transferred to the selection medium (MS with Kanamycin). The explants were kept on this medium for 3 weeks to allow the formation of calli and shoots from the transfected cells. The kanamycin resistant shoots were transferred into the rooting MS medium. Rooted plantlets were grown to maturity in the greenhouse. Integration of the transgene was verified by PCR amplification of a NiR-GUS fragment from genomic DNA. The primer used were:

- Nir-102c – 5' CACACTTCTTCACTCACCTCTCAA 3' (SEQ ID NO:25)
Nir-2016c – 5' ATCTAGGAGGGGCAGACATTG 3' (SEQ ID NO:26)
GUS228r – 5' TCGGTATAAAGACTTCGCGCTGAT 3' (SEQ ID NO:27)

Nitrate induction

Transgenic and non-transgenic tobacco and alfalfa plants were grown in vermiculite medium without nitrate. Mineral balance was kept by repeated additions of nitrate-free Hoagland's solutions (Hoagland and Vamon, 1950, Circular 347, *California Agr. Exp. Stat. Berkeley*). Nitrate induction was performed by watering the plants with 20-20-20 fertilizer at a concentration of 5gL⁻¹ or as an alternative with Hoagland's solution supplemented with 40 mM nitrate.

NiR promoter activity in tobacco leaves

The NiR derived promoters were placed upstream of the GUS reporter gene in transcriptional and translational fusions. The 5' deletions of the NiR promoter analyzed here consisted in (1) a putative full length promoter comprising 2813 bp upstream of the initial ATG of the coding region, (2) a 1905 bp version of the promoter, and (3) a shorter 1111 bp version of the promoter. The 3' end of the promoter was fused to the 5' end of the GUS coding region to form transcriptional and translational

fusions. Translational fusions analyzed allowed the production of β -glucuronidase containing (1) the NiR chloroplast transit peptide, (2) the NiR transit peptide with an additional 17 amino acids from NiR, and (3) the NiR transit peptide with an additional 36 amino acids from NiR. The twelve combinations of 5' and 3' deletions of the NiR promoter introduced into tobacco plants are presented in Fig. 1.

The gene constructs were transferred into tobacco plants using the *Agrobacterium*-mediated transfection method (Khouidi et al., 1997, *Gene* 197:343-351). Transgenic plants were transferred to growth chambers and analyzed for their leaf β -glucuronidase content before and after nitrate fertilization. Fig. 1 presents the median level of β -glucuronidase activity measured in the 1st expanded leaf of plantlets.

All the NiR derived promoters showed reactivity to nitrate induction. Between 5 and 10 fold increase of β -glucuronidase expression was generally observed, irrespectively of the promoter truncation, indicating that important nitrate responsive elements are contained within the first 1.1 kb upstream of the initial ATG. Both 5' and 3' deletions of the NiR promoter led to important modifications of β -glucuronidase activity. The highest level of GUS expression was obtained with the 2.8 kb promoter, indicating that the far upstream regions have a regulatory role for the level of NiR expression in the leaves.

The translational fusions of the promoter to the GUS coding region resulted in variable expression level depending on the extension of the 5' end of the promoter. However, the shortest fusion (containing the 17 a.a. NiR transit peptide fused to the amino-terminal end of the β -glucuronidase) constantly resulted in the highest level of activity for all three 5' end truncations. This short translational fusion, combined with the longest extension of upstream promoter regions gave rise to the strongest promoter (3kb+50). When induced, this specific construct resulted in more than 13-fold the level of GUS expression obtained with the 35S-GUS-NOS construct.

When considering the longest 5' extension of the promoter, the transcriptional fusion to the GUS gene (3kb-5) was ~7 times less effective than the short translational fusion (3kb+50). However, in its induced state, the level of GUS expression in the plants harboring the 3kb-5 promoter deletion was more than 1,8-fold that observed in the 35S-GUS-NOS plants.

Taken together, the results presented here clearly indicate that the sequences upstream of the alfalfa NiR gene have the capacity to drive high and inducible expression of an exogenous gene in tobacco leaves.

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Efficiency of NiR terminator

Tobacco plants were transformed with constructs consisting of promoter NiR and deletions (SEQ ID NOS: 2, 3, 5 and 6), and 35S, together with 3'UTR sequences and terminator (SEQ ID NOS: 15 and 16), functionally positioned to drive transcription and termination of reporter gene GUS. Growth, nitrate induction and GUS activity measurements were performed as per experiment illustrated in Fig 1. Results shown in Fig 2 demonstrate that the terminator sequence of Nir allows termination of transcription into a translatable messenger RNA.

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NiR promoter activity in alfalfa leaves

Transgenic alfalfa plant containing the gene constructs presented in Fig. 3 were obtained using the *Agrobacterium*-mediated transfection method of Desgagnés et al. (1995, *Plant Cell Tissue Organ Cult.* 42:129-140). The *in vitro* plants were transferred into growth chamber to allow a normal vegetative growth. Cuttings from each transgenic line were grown in vermiculite and fertilized with nitrate-free Hoagland medium. After two weeks, the roots were inoculated with Nitragin (LiphaTech inc., Milwaukee, WI). Two weeks after inoculation, nodules had developed on the roots. Nodulated plants were allowed to continue their vegetative growth for at least another week before the fluorometric measurement of β -glucuronidase activity (before induction)

30

was performed. After the measurement, the plants were watered with Hoagland medium containing 40 mmol KNO₃. Two days after induction, leaf β -glucuronidase activity was measured to evaluate the nitrate inducibility of the NiR promoter in alfalfa leaves. Results are presented in Fig. 3. Results show that promoter NiR induces expression of GUS reporter gene upon addition of nitrate in nodulated alfalfa plants. Taken together, this last series of result demonstrate that NiR promoter inducibility can be used to positively regulate expression of a foreign gene in alfalfa plants when fixation of atmospheric nitrogen is replaced by nitrate assimilation.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for regulating transcription of a foreign gene in transgenic organisms, comprising the steps of:
 - a) preparing a transgenic organism using an expression construct consisting of at least a nitrogen-inducible promoter having a sequence selected from the group consisting of SEQ ID NOS: 1 to 13 and functional fragments and derivatives thereof, and an ORF of a gene, wherein said promoter is operationally located with respect to said gene for expression of said gene.
2. The method of claim 1, which further comprises the step of regulating transcriptional expression of said gene by addition or removal of a nitrogen inducer.
3. A method for regulating transcription of a foreign gene in transgenic organisms comprising:
 - a) preparing an expression construct consisting of at least a nitrogen-inducible promoter with or without cis-acting sequence, an ORF of a gene, and a polyadenylation signal end site at the 3'end of said construct, wherein said promoter is operationally located with respect to said gene for expression of said gene and modulated for transcriptional expression of said gene by addition or removal of a nitrogen inducer;
 - b) sub-cloning the construct of step a) into a suitable transfection vector for said organism;
 - c) transferring said vector into DNA of said organism or a cell thereof; and
 - d) selecting for transgenicity on a suitable medium.
4. The method of claim 3, wherein said organism is a plant.
5. The method of claim 4, which further comprises the steps of:
 - e) introducing the vector into a suitable *Agrobacterium tumefaciens* strain;

- f) using the *Agrobacterium* strain of step a) to transfer T-DNA into a plant cell;
- g) selecting for transgenicity of said plant cell on a suitable medium;
- h) regenerating embryos or plantlets from said transgenic cells; and
- i) growing mature plants from said regenerated embryos.

6. The method of claim 3, wherein the cis-acting sequence is isolated from 5' upstream region of an expressed Nir gene in alfalfa.

7. The method of claim 3, wherein the promoter has the sequence set forth in SEQ ID NOS:1 to 13 and functional fragments and derivatives thereof.

8. The method of claim 1, wherein the organism is selected from the group consisting of a plant, a fungus, a bacteria, a yeast and an animal.

9. The method of claim 8, wherein the plant is a dicot.

10. The method of claim 9, wherein the dicot is alfalfa or tobacco.

11. The method of claim 2, wherein the nitrogen inducer is nitrate.

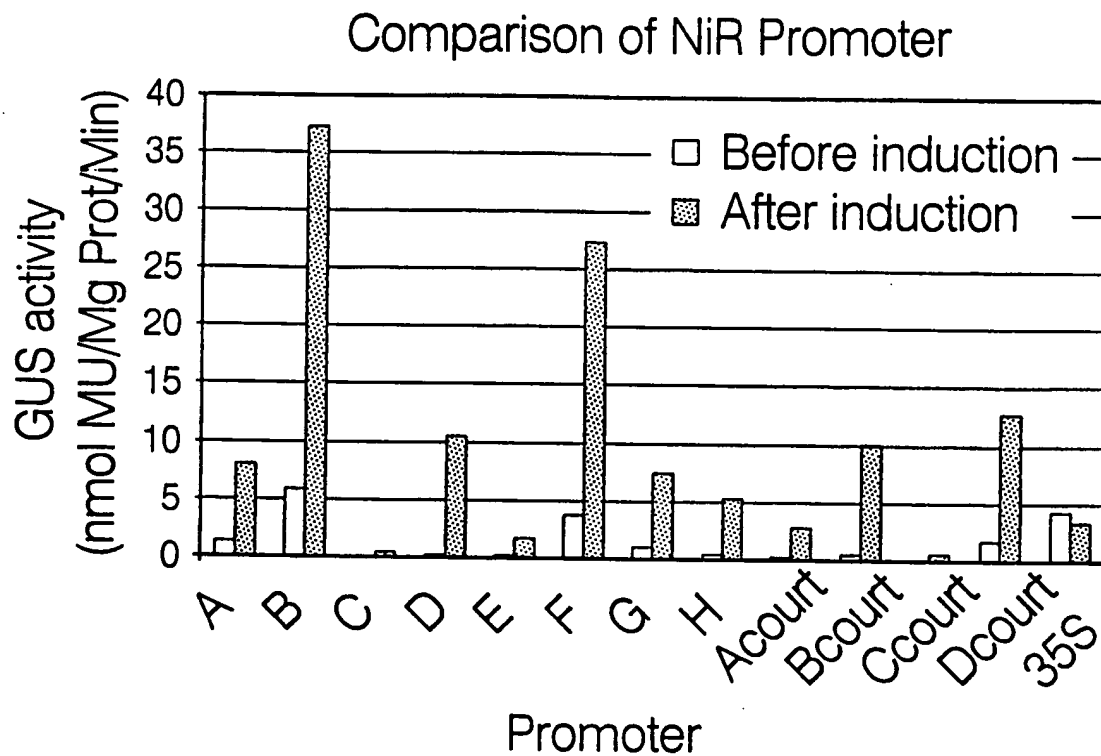
12. The method of claim 3, wherein the DNA transfer method is any suitable transfer method including DNA bombardment, electroporation, PEG-mediated DNA transfer and whiskers.

13. The method of claim 3, wherein the expression construct comprises at least a nitrogen-inducible promoter and at least one cis- or trans-acting elements.

14. The method of claim 3, wherein the promoter or cis-acting sequence is isolated from the 5' upstream region of any gene involved in a nitrogen assimilatory pathway.
15. The method of claim 3, wherein the promoter or cis-acting sequence is isolated from the 5' upstream region of any gene for which transcription is modulated by availability of a nitrogen source.
16. The method of claim 3, wherein the promoting or cis-acting sequence is any sequence for which transcriptional activity is modulated by addition or removal of any nitrogen source in or from any living organism's environment.
17. The method of claim 3, wherein the organism from which the promoter or cis-acting sequence is isolated from is any plant, fungus, yeast, bacteria or animal.
18. A promoter for promoting transcription of a foreign gene in transgenic organisms, which comprises a nitrogen-inducible promoter with or without cis-acting sequence for expression of said gene and adapted to be modulated for transcriptional expression of said gene by addition or removal of a nitrogen inducer.
19. The promoter of claim 18, wherein said promoter has a sequence selected from the group consisting of SEQ ID NOS: 1 to 13 and functional fragments and derivatives thereof.
20. A terminator allowing expression of a foreign gene in transgenic organisms being used in combination with a promoter, which comprises a polyadenylation signal end site for insertion at the 3' end of said gene, wherein said terminator is operationally located with respect to said gene and said promoter and thereby allows expression of said gene.

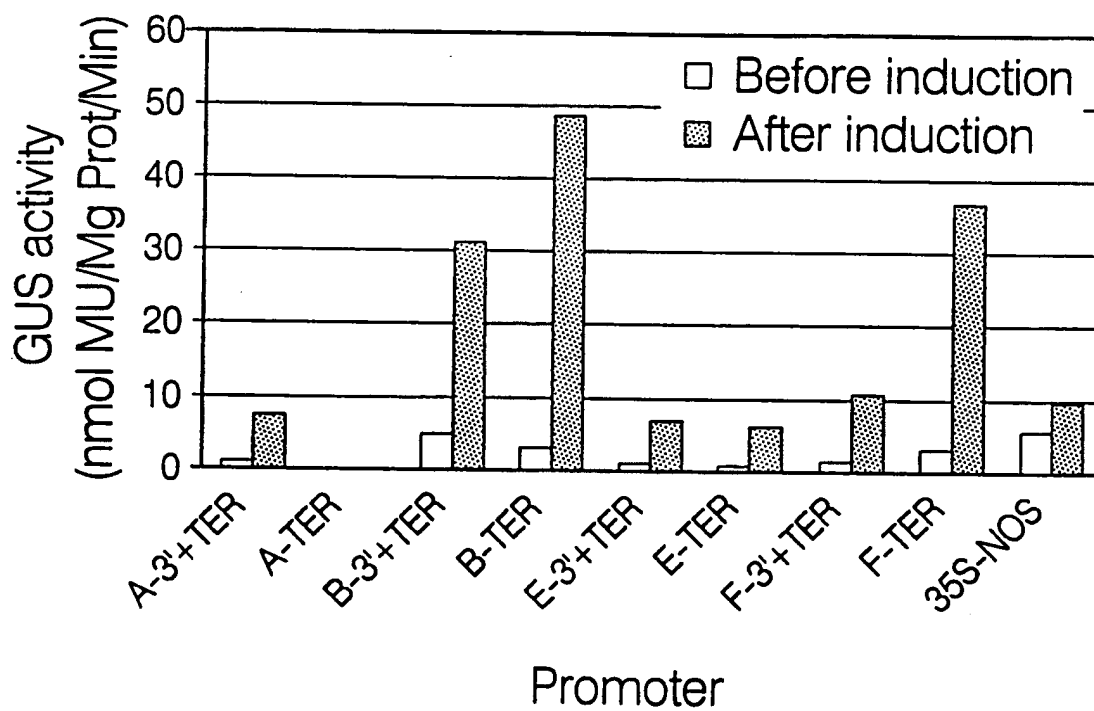
21. The terminator of claim 19, wherein said terminator has a sequence selected from the group consisting of SEQ ID NOS: 14 to 16 and functional fragments and derivatives thereof.

1 / 3

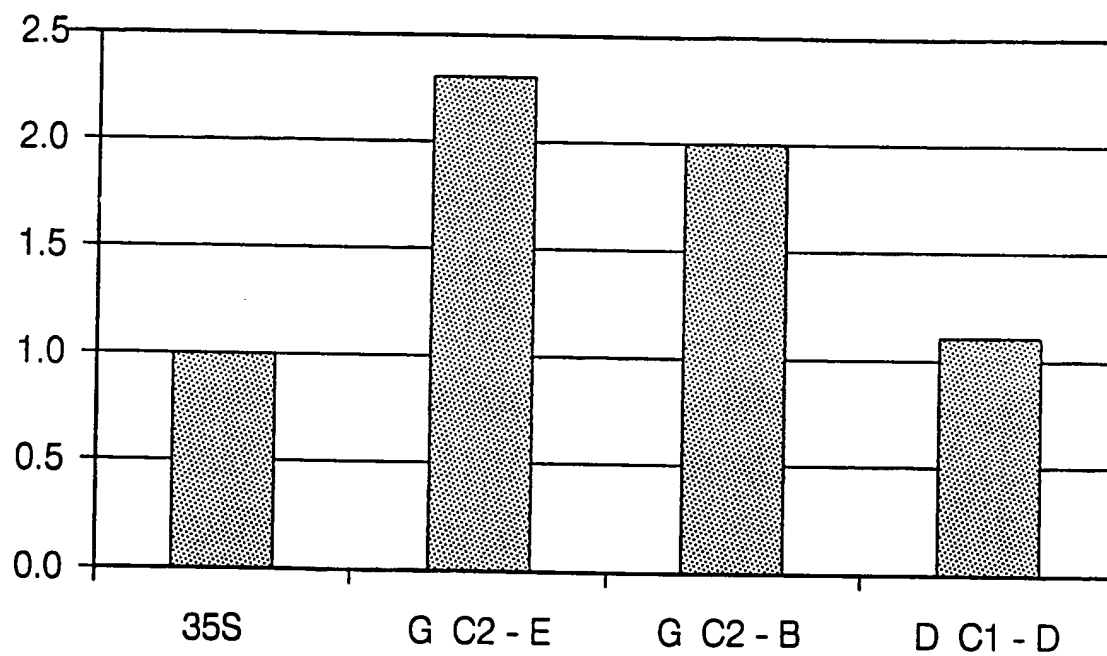
*Fig. 1*

2 / 3

Comparison of NiR Promoter

*Fig. 2*

3 / 3

*Fig. 3*

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D'AOUST, Marc-André
MEDICAGO INC.

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acatgtcatt	tattttgcct	cactaactca	attttgcata	taacaatgat	aagtgatatt	1560
ttgactcaca	aaattttacat	caaatttcga	catcgtttat	tatgttcatt	ggatgattaa	1620
caaatataac	aaactttgca	actaattaac	caccaactga	atataattaa	ctataactgt	1680
gaaagtagtt	aaccatattt	tttagatgta	tatatcatcc	gttgaatgta	attattcata	1740
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gacgaaaatg	taatgagact	ttaatgttga	ctttgacacc	gcaccacatg	tgcccttttac	1860
atatagttta	tatgacaagt	aatgacaatc	cttgctctat	tataaggcga	cccttagctc	1920
caaccaaagg	acgatggagt	taagaaagaa	actcttgctt	acttgtaagg	tccacacttc	1980
ttcactcacc	tctcaatttc	atcctacaaa	aatgtccaaa	cttctctttc	tcacaatcac	2040
aaactcattc	caaacacact	ctcttctcc				2069

<210> 4

<211> 1220

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 4

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tactattatt	ttattttattg	tgtttttctt	ttttctaccg	gaacttttta	gaacggatcc	120
caactcgttc	cggggccgct	acaactgaaa	caaaagaaga	tattttctct	ctcttcagaa	180
atgtaagttt	tccttttacag	atacccttcc	accatttgat	tcagatgtgg	tgactagaga	240
taaagcatac	taatttgact	cttggaacc	cataaagttt	atgttatccg	tggtctggac	300
caatccactt	gggggcataa	cctgtgtcta	tgtgtgggtt	ggtttccatt	ctgatttatg	360
cggcgacttg	taatttaaaa	tctaggaggg	gcagacattg	aacaatccca	atattttaat	420
aacttatgca	agattttttt	tattaatgag	atgatgtgtt	tgtgactgag	attgagtcac	480
acatttcact	aagaaatggg	tccaagtacc	aaactatcat	gaccagttg	caaacatgac	540
gttcgggagt	ggtcactttg	atagttcaat	ttcatcttgg	cttcttattc	cttttataat	600
tctaattctt	cttgtgtaaa	ctatttcatg	tattattttt	ctttaaaatt	tacatgtcat	660
ttattttgcc	tcactaactc	aattttgcat	ataacaatga	taagtgatat	tttgactcac	720
aaaattttaca	tcaaatttcg	acatcgttta	ttatgttcat	tggatgatta	acaaatataa	780
caaactttgc	aactaattaa	ccaccaactg	aatataatta	actataactg	tgaaagtagt	840
taacctatct	tttttagatgt	atatatcatc	cgttgaatgt	aattattcat	atatttgaac	900
taagttatcc	tacaacttaa	agaacttaaa	gaactcgggt	tgagacctgg	ggacgaaaat	960
gtaatgagac	tttaagtgtg	actttgacac	cgcaccacat	gtgcctttta	catatagttt	1020
atatgacaag	taatgacaat	ccttgctcta	ttataaggcg	acccttagct	ccaaccaaag	1080
gacgatggag	ttaagaaaga	aactcttgct	tacttgtaag	gtccacactt	cttcaactc	1140
ctctcaattt	catcctacaa	aaatgtccaa	acttctcttt	ctcacaatca	caaactcatt	1200
ccaaacacac	tctcttctcc					1220

<210> 5

<211> 2863

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 5

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agaagagccc	agacttcaag	aagcgacctt	gcaagtgcac	tcgaggggtca	gaaactgtat	120
atcatatcta	tgtgagagaa	aggggaacat	ttgagatgga	gtccattttac	ttgaggtata	180

cttattattt	caataa	atttgtatac	ttcttattta	gatttcaaa	tttgtcatta	240
agctataatc	caaaataaat	tacgatcaaa	tatgcaaag	ttagccagta	cttgtgttaa	300
acttgatggc	atctcttggg	ttctttggca	atcacatgcc	taagaaataa	atagtatcat	360
atgattgtgt	ttggtcagac	ttcagagtca	gatgactctg	tttggataaa	cagcttaatt	420
aagcgcttat	agaatatcat	atgattgtgt	ttggtcagac	ttcagagcat	ctcttggttt	480
ctctggcaat	catatgccta	agaaataaat	agtatcatat	gattgtgttt	ggtcagactt	540
cagagtcaga	tgacctgtgt	tgggtaaaca	gcttaattaa	gtgcttatag	aataagcgct	600
tatcatataa	gtgcttttgt	acagttattt	ctatgaaagt	agaagaaata	gtcatattgt	660
tttaatatata	gctatcctgg	agagcttgtg	gaaataacca	gaaaagaact	tatggacacg	720
tcagagctg	tttacataag	atctccctaa	cagtctcaaa	agtgtttatg	ccagtagata	780
aattcaaata	agtcaatcta	aacagaccct	aaatccatta	tggtagctat	catttttagct	840
tattccatct	ttattaagaa	tgtcatgaga	taacataatg	ataacacatt	attttgacac	900
aatgggag	atctagcaat	ttaactctgg	agtccttcaa	gactgctgtt	cttacgaagt	960
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gcgataaatt	gaagggtttac	aaaatatacc	ctgcgggctt	gacacagagg	caagctcttt	1080
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ctagcattag	ctctttcact	taactgagag	aatgaagttt	taggaatgag	tatgacctg	1260
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catatttaat	gttgggtgtg	agtgttttta	cttgattata	tccagacagt	tacaagttgg	1440
acaacaagat	tgtgggtctg	tactgttatt	tattttattt	tttttttagca	gaaacacctt	1500
atcttttgtt	tcgtttgaat	gtagaatgaa	aataaaaagaa	agaaaatata	acatcatcgg	1560
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aaagaagata	ttttctctct	cttcagaaat	gtaagttttc	ctttacagat	acccattcac	1800
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agacattgaa	caatcccaat	attttaataa	cttatgcaag	atttttttta	ttaatgagat	2040
gatgtgtttg	tgactgagat	tgagtcatac	atttcactaa	gaaatgggtc	caagtaccaa	2100
actatcatga	cccagttgca	aacatgacgt	tcgggagtg	tcactttgat	agttcaattt	2160
catcttggct	tcttattcct	tttataattc	taattcttct	tgtgtaaaact	atttcatgta	2220
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aacaatgata	agtgatattt	tgactcacia	aattttacatc	aaatttcgac	atcgtttatt	2340
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caccacattg	gccttttaca	tatagtttat	atgacaagta	atgacaatcc	ttgctctatt	2640
ataaggcgac	ccttagctcc	aaccaaagga	cgatggagtt	aagaaagaaa	ctcttgctta	2700
cttgtaaggt	ccacacttct	tcactcacct	ctcaatttca	tcctacaaaa	atgtccaaac	2760
ttctctttct	cacaatcaca	aactcattcc	aaacacactc	tcttctccaa	aaatgtcttc	2820
cttctcagta	cgtttctctca	ccccaccatc	catctctcgt	ccc		2863

<210> 6

<211> 2124

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 6

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aaacagagccc	taaattccatt	atgggtaccta	tcatttttagc	ttattccatc	tttattaaga	120
atgtcatgag	ataacataat	gataacacat	tatttttgaca	caaattgggca	gatctagcaa	180
tttaactctg	gagtccttca	agactgctgt	tcttacgaag	ttcacgtccc	tgaatcatgt	240

tcctgtatgg	atgaaa	gacctcaa	at	tctaaaaggt	ggcg	aat	tgaaggttta	300
caaaatatac	cctgcgggct	tgacacagag	gcaagctcct	tataccttcc	agttcaacgg			360
ggatgttgat	ttcagaagtc	acttgagag	caatccttgt	gccaggttg	aagtaatttt			420
tgtgtagcat	atgttgagct	acctacaatt	tacatgatca	cctagcatta	gctctttcac			480
ttaactgaga	gaatgaagtt	ttaggaatga	gtatgaccat	ggagtcggca	tggctttgta			540
atgcctaccc	tactttggcc	aactcatcgg	ggatttacat	tcagaaaata	tacatgactt			600
caaccatact	taaaccctt	tttgtaagat	aactgaatgt	tcataattta	tgttgggttg			660
tagtggtttt	acttgattat	atccagacag	ttacaagttg	gacaacaaga	ttgtgggtct			720
gtactgttat	ttatttattt	tttttttagc	agaaacacct	tatcttttgt	ttcgtttgaa			780
tgtagaatga	aaataaaaaga	aagaaaatat	aacatcaatc	gccgcgcttg	tctaatttcg			840
ggcagttagg	atcctctccg	gtcaccggaa	agtttcagta	gaagaaacaa	aacaccgtga			900
ctaaaatgat	actattattt	tatttattgt	gtttttcttt	tttctaccgg	aacttttttag			960
aacggatccc	aactcggttc	ggggcgctta	caactgaaac	aaaagaagat	attttctctc			1020
tcttcagaaa	tgtaagtttt	cctttacaga	taccatttca	ccatttgatt	cagatgtggg			1080
gactagagat	aaagcatact	aatttgactc	ttggaaaccc	ataaagttta	tgttatccgt			1140
gttctggacc	aatccacttg	ggggcataac	ctgtgtctat	gtgtgggttg	gtttccattc			1200
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tattttaata	acttatgcaa	gatttttttt	attaatgaga	tgatgtgttt	gtgactgaga			1320
ttgagtcata	catttccacta	agaaatgggt	ccaagtacca	aactatcatg	accagttgc			1380
aaacatgacg	ttcgggagtg	gtcactttga	tagttcaatt	tcactcttggc	ttcttattcc			1440
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gaaagttagt	aaccatattt	tttagatgta	tatatcatcc	gttgaatgta	attattcata			1740
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gacgaaaatg	taatgagact	ttaatgttga	ctttgacacc	gcaccacatg	tgccttttac			1860
atatagttta	tatgacaagt	aatgacaatc	cttgctctat	tataaggcga	ccccttagctc			1920
caaccaaagg	acgatggagt	taagaaagaa	actcttgctt	acttgtaagg	ttcacacttc			1980
ttcactcacc	tctcaatttc	atcctacaaa	aatgtccaaa	cttctctttc	tcacaatcac			2040
aaactcattc	caaacacact	ctcttctcca	aaaatgtctt	ccttctcagt	acgtttcttc			2100
acccaccat	ccatctctcg	tccc						2124

<210> 7

<211> 1160

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 7

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cagaaatgta	agttttcctt	tacagatacc	cattcaccat	ttgattcaga	tgtgggtgact	120
agagataaag	catactaatt	tgactcttgg	aaacccataa	agtttatgtt	atccgtgttc	180
tggaccaatc	cacttggggg	cataacctgt	gtctatgtgt	ggtttggttt	ccattctgat	240
ttatgcggcg	acttgtaatt	taaaatctag	gaggggcaga	cattgaacaa	tcccaatatt	300
ttaataactt	atgcaagatt	ttttttatta	atgagatgat	gtgtttgtga	ctgagattga	360
gtcatacatt	tcactaagaa	atggttccaa	gtaccaaact	atcatgaccc	agttgcaa	420
atgacgttcg	ggagtggtca	ctttgatagt	tcaatttcat	cttggttctt	tattcctttt	480
ataattctaa	ttcttcttgt	gtaaactatt	tcatgtatta	tttttcttta	aaattttacat	540
gtcatttatt	ttgcctcact	aactcaattt	tgcatataac	aatgataagt	gatattttga	600
ctcacaaaat	ttacatcaaa	tttcgacatc	gtttattatg	ttcattggat	gattaacaaa	660
tataacaaac	tttgcaacta	attaaccacc	aactgaatat	aattaaactat	aactgtgaaa	720
gtagttaacc	atatttttta	gatgtatata	tcattccgttg	aatgtaatta	ttcatatatt	780
tgaactaagt	taccctacaa	cttaaagaac	ttaaagaact	cggtttgaga	cctggggacg	840
aaaatgtaat	gagacttta	tgttgacttt	gacaccgcac	cacatgtgcc	ttttacatat	900
agtttatatg	acaagtaatg	acaatccttg	ctctattata	aggcgaccct	tagctccaac	960
caaaggacga	tggagttaag	aaagaaactc	ttgcttactt	gtaagggtcca	cacttcttca	1020

ctcacctctc	aaacatcc	tacaaaaatg	tccaaacttc	tcttcac	aatcacaaac	1080
tcattccaaa	cacactctct	tctccaaaaa	tgtcttcctt	ctcagtagct	ttcctcaccc	1140
caccatccat	ctctcgtccc					1160

<210> 8

<211> 2904

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 8

ctgtacattc	atcttgccgc	ctttgcattc	acttggccac	aaagagtaga	gagaaggaag	60
agaagagccc	agacttcaag	aagcgacctt	gcaagtgcac	tcgaggggtca	gaaactgtat	120
atcatatcta	tgtgagagaa	aggggaacat	ttgagatgga	gtccatttac	ttgaggtata	180
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agctataatc	caaaataaat	tacgatcaaa	tatgcaaagt	ttagccagta	cttgtgttaa	300
acttgatggc	atctcttggt	ttctttggca	atcacatgcc	taagaaataa	atagtatcat	360
atgattgtgt	ttggtcagac	ttcagagtca	gatgactctg	tttggataaa	cagcttaatt	420
aagcgcttat	agaatatcat	atgattgtgt	ttggtcagac	ttcagagcat	ctcttggttt	480
ctctggcaat	catatgccta	agaaataaat	agtatcatat	gattgtgttt	ggtcagactt	540
cagagtcaga	tgaccctggt	tgggtaaaca	gcttaattaa	gtgcttatag	aataagcgct	600
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tattccatct	ttattaagaa	tgtcatgaga	taacataatg	ataacacatt	attttgacac	900
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tgtgggtttg	tttccattct	gatttatgcg	gcgacttgta	atttaaaatc	taggaggggc	1980
agacattgaa	caatcccaat	attttaataa	cttatgcaag	atttttttta	ttaatgagat	2040
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actcggtttg	agacctgggg	acgaaaatgt	aatgagactt	taatgttgac	tttgacaccg	2580
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ataaggcgac	ccttagctcc	aaccaaaagga	cgatggagtt	aagaaagaaa	ctcttgctta	2700
cttgtaaggt	ccacacttct	tcactcacct	ctcaatttca	tcctacaaaa	atgtccaaac	2760

ttctctttct	ca	tcaca	aactcattcc	aaacacactc	tcttc	caa	aatgtcttc	2820
cttctcagta	cg	tttcctca	ccccaccatc	catctctcgt	cccaacaaaa	catggctact	2880	
atctgctgca	actccatcag	ttgc					2904	

<210> 9

<211> 2165

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 9

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aaacagaccc	taaatccatt	atggtacct	tcatttttagc	ttattccatc	tttattaaga	120
atgtcatgag	ataacataat	gataacacat	tattttgaca	caaattgggca	gatctagcaa	180
tttaactctg	gagtccttca	agactgctgt	tcttacgaag	ttcacgtccc	tgaatcatgt	240
tcctgtatgg	aagcctgaaa	gacctcaaat	tctaaaagggt	ggcgataaat	tgaagggtta	300
caaaatatac	cctgcgggct	tgacacagag	gcaagctctt	tataccttcc	agttcaacgg	360
ggatgttgat	ttcagaagtc	acttgagag	caatccttgt	gccaagtttg	aagtaatttt	420
tgtgtagcat	atgttgagct	acctacaatt	tacatgatca	cctagcatta	gctctttcac	480
ttaactgaga	gaatgaagtt	ttaggaatga	gtatgaccat	ggagtcggca	tggttttgta	540
atgcctaccc	tactttggcc	aactcatcgg	ggattttacat	tcagaaaata	tacatgactt	600
caaccatact	taaaccctt	tttgtaagat	aactgaatgt	tcataattta	tggtgggttg	660
tagtgttttt	acttgattat	atccagacag	ttacaagttg	gacaacaaga	ttgtgggtct	720
gtactgttat	ttattttattt	tttttttagc	agaaacacct	tatcttttgt	ttcgtttgaa	780
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acccaccat	ccatctctcg	tcccaacaaa	acatggctac	tatctgctgc	aactccatca	2160
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<210> 10

<211> 1316

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 10

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caactcggtc	cggggccgct	acaactgaaa	caaaagaaga	tattttctct	ctcttcagaa	180
atgtaagttt	tcctttacag	ataccattc	accatttgat	tcagatgtgg	tgactagaga	240
taaagcatac	taatttgact	cttggaacc	cataaagttt	atgttatccg	tgttctggac	300
caatccactt	gggggcataa	cctgtgtcta	tgtgtggttt	ggtttccatt	ctgatttatg	360
cggcgacttg	taatttataa	tctaggaggg	gcagacattg	aacaatcca	atattttaat	420
aacttatgca	agattttttt	tattaatgag	atgatgtgtt	tgtgactgag	attgagtcac	480
acatttcact	aagaaatggt	tccaagtacc	aaactatcat	gacccagttg	caaacatgac	540
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tctaattctt	cttgtgtaaa	ctatttcatg	tattattttt	ctttaaaatt	tacatgtcat	660
ttattttgcc	tcactaactc	aattttgcat	ataacaatga	taagtgatata	tttgactcac	720
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ccaaacacac	tctcttctcc	aaaaatgtct	tccttctcag	tacgtttcct	cacccaccca	1260
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<210> 11

<211> 2971

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 11

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aagcgcttat	agaatatcat	atgatttgtg	ttggtcagac	ttcagagcat	ctcttggttt	480
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cagagtcaga	tgaccctgtt	tgggtaaaaca	gcttaattaa	gtgcttatag	aataagcgct	600
tatcatataa	gtgcttttgt	acagttattt	ctatgaaagt	agaagaaata	gtcatattgt	660
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atctgctgca	actccatcag	ttgcacctgt	ttcaacacca	caagttgatg	catcaaggtt	2940
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<210> 12

<211> 2232

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 12

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atgtcatgag	ataacataat	gataacacat	tatttttgaca	caaattgggca	gatctagcaa	180
tttaactctg	gagtccttca	agactgctgt	tcttacgaag	ttcacgtccc	tgaatcatgt	240
tcttgatagg	aagcctgaaa	gacctcaaat	tctaaaagg	ggcgataaat	tgaaggttta	300
caaaatatac	cctgcgggct	tgacacagag	gcaagctctt	tataccttcc	agttcaacgg	360
ggatgttgat	ttcagaagtc	acttgagag	caatccttgt	gccaaagttg	aagtaatttt	420
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acccaccat	ccatctctcg	tccaacaaa	acatggctac	tatctgctgc	aactccatca	2160
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aaagatgggt	ac					2232

<210> 13

<211> 1383

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 13

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caactcgttc	cggggccgct	acaactgaaa	caaaaagaaga	tattttctct	ctcttcagaa	180
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taaagcatac	taatttgact	cttggaacc	cataaagttt	atgttatccg	tggtctggac	300
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tac						1383

<210> 14

<211> 3472

<212> DNA

<213> Artificial Sequence

<220>

<223> Sequences to be used as terminator for regulating expression of foreign genes

<400> 14

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aaggaagagt	atagaggagg	tattaatcct	caggagaaag	ttaagattca	gaaagaacct	120
atgaagcttt	ttatggaagg	tggtattaat	gatttggcta	atatgtctct	tgaagagatt	180
gaaagctcta	agcttactaa	agatgatatt	gatgttagac	ttaaatggct	tggctctttt	240
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tcgtaatttg	tcatttgaga	gctacggaga	tcgttgagtt	tacgtatgag	tgttttagtct	300
cacattaatt	atgaatggtc	aaaatgttaa	atttataaga	gatgtaatct	atatacctaa	360
tgcattaaaa	atttggtatg	agatgcgacg	ccccctttt	ttgtggtcct	gaagtataga	420
cttgtttgtcg	cttctggtgc	actctcatac	ttcccaacaa	ggagaaaaaa	ctaccataac	480
aattaacaaa	ctaacatttg	ttattttaaaa	aaacatacgg	atactgtttt	ttccccattt	540
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(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number
WO 01/25454 A3(51) International Patent Classification⁷: C12N 15/82, 9/02

(21) International Application Number: PCT/CA00/01143

(22) International Filing Date: 2 October 2000 (02.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/157,133 4 October 1999 (04.10.1999) US(71) Applicant (for all designated States except US): MED-
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Suite 1600, 1981 McGill College Avenue, Montréal,
Québec H3A 2Y3 (CA).(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

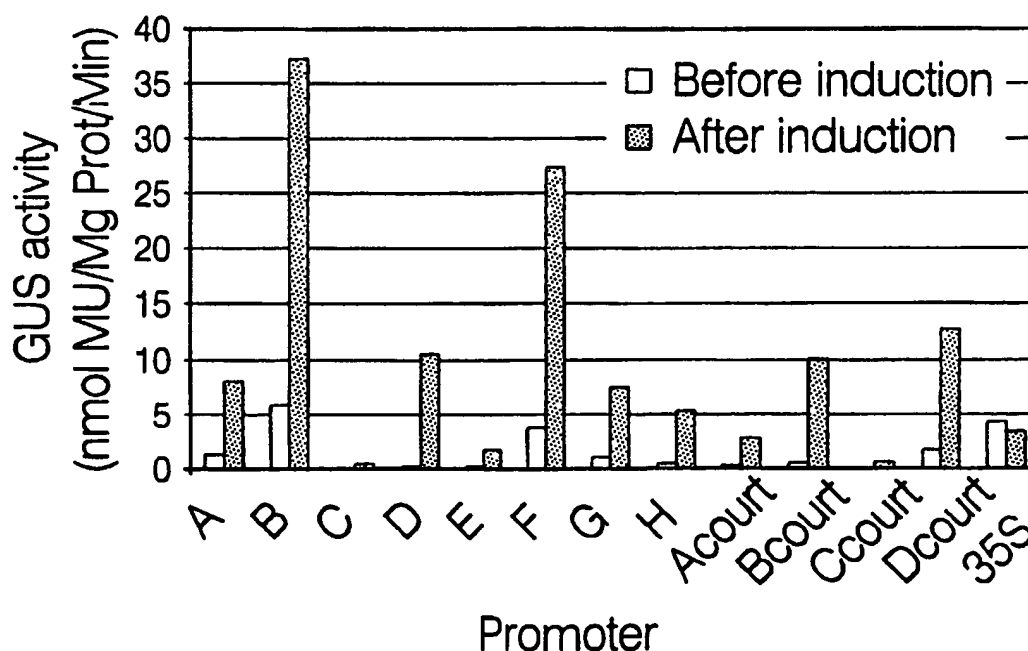
— with international search report

(88) Date of publication of the international search report:
8 November 2001

[Continued on next page]

(54) Title: METHOD FOR REGULATING TRANSCRIPTION OF FOREIGN GENES IN THE PRESENCE OF NITROGEN

Comparison of NiR Promoter



(57) Abstract: The present invention relates to a method of regulating the transcription of transgene in genetically-modified organisms. More specifically, the invention relates to the use of expression vectors harboring the coding sequence of a gene of interest under the transcriptional control of promoting sequences for which activity is regulated by the presence of nitrogen.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12N15/82 C12N9/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BACK E ET AL: "ISOLATION OF THE SPINACH NITRITE REDUCTASE GENE PROMOTER WHICH CONFERS NITRATE INDUCIBILITY ON GUS GENE EXPRESSION IN TRANSGENIC TOBACCO" PLANT MOLECULAR BIOLOGY, vol. 17, no. 1, 1991, pages 9-18, XP002167603 ISSN: 0167-4412 cited in the application the whole document</p> <p style="text-align: center;">--- -/--</p>	1-5, 7-21

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Date of the actual completion of the international search

6 June 2001

Date of mailing of the international search report

19/06/2001

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PALAUQUI' JEAN-CHRISTOPHER ET AL: "Frequencies, timing, and spatial patterns of co-suppression of nitrate reductase and nitrite reductase in transgenic tobacco plants." PLANT PHYSIOLOGY (ROCKVILLE), vol. 112, no. 4, 1996, pages 1447-1456, XP002167604 ISSN: 0032-0889 the whole document</p> <p>---</p>	1-5,7-21
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X	<p>US 4 997 930 A (ROTHSTEIN STEVEN J ET AL) 5 March 1991 (1991-03-05) the whole document</p> <p>-----</p>	1-4,7,8, 11,14-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 00/01143

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4997930 A	05-03-1991	NONE	
